Agrobacterium-Mediated Transformation of *Cry1Ac* Gene to Tobacco (*Nicotiana tabacum*) and Evaluation of *Heliothis armigera* Resistance

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Abstract: *Agrobacterium* mediated gene transfer is a standard technique in plant genetic engineering. For the gene transfer, leaf discs of tobacco variety K326 were co-cultured with *A. tumefaciens* strain EHA carried *Cry1Ac, bar* and GUS genes which are insect toxin, herbicide resistant and reporter genes respectively in the plasmid pITB2. Phosphinothricin (PPT)-resistant shoots that express GUS activity were derived from the cultured leaf discs and the presence of *Cry1Ac* was checked using PCR and toxin protein was determined by Western Blot analysis. The *in vivo* assay using cotton bollworm larvae showed that the transgenic plants were protected from the feeding and the loss became minimized.

Keywords: Tobacco transformation, insecticidal proteins, transgenic plants, *Bacillus thuringiensis* toxin

บทคัดย่อ: การถ่ายยีนเข้าสู่พืชโดยผ่านทางเชื้อ *Agrobacterium* เป็นที่ยอมรับในกระบวนการทางพันธุวิศวกรรมทางด้านพืช ในการทดลองนี้ใช้ชิ้นส่วนของใบยาสูบพันธุ์ K326 มาเลี้ยงร่วมกับ *A. tumefaciens* สายพันธุ์ EHA ที่มีพลาสมิด pITB2 ที่ได้รับการสอดถ่ายยีน *Cry1Ac, bar* และ GUS ซึ่งเป็นยีนที่ควบคุมการสร้างสารพิษต่อแมลง ยีนต้านทานต่อสารปราบวัชพืชและยีนรายงาน หรือใช้ตรวจสอบตามลำดับ หลังจากการเลี้ยงชิ้นใบยาสูบในอาหาร พบว่ายอดที่มีลักษณะต้านทานต่อสารปราบวัชพืช phosphinothricin และมีการแสดงผลของ GUS ยีนพัฒาจากส่วนของชิ้นใบ เมื่อนำยอดที่ได้มาตรวจสอบการแสดงออกของยีน *Cry1Ac* โดยวิธี Western Blot และการนำหนอนเจาะสมอฝ้ายมาเลี้ยงบนใบยาสูบที่ได้รับการถ่ายยีน ผลการทดลองแสดงว่า ยาสูบที่ได้รับการถ่ายยีนสามารถป้องกันการกัดกินของหนอนได้ทำให้ความเสียหายอันเนื่องจากการทำลายของหนอนลดลงอย่างชัดเจน

คำสำคัญ: พันธุวิศวกรรมใบยาสูบ โปรตีนฆ่าแมลง พืชดัดแปลงพันธุกรรม สารพิษจาก *Bacillus thuringiensis*

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Introduction

In recent studies of plant biotechnology, tobacco has been used as a model crop in the development of new technologies. Tobacco is an excellent material for model experiments on genetic transformation. With advances in gene technology, more and more genes have been transferred to plant for various purposes. A number of useful genes have been introduced into the tobacco genome to produce new traits for the tobacco varieties improvement. Some of the genes are related to insect resistant, herbicide resistant to and tolerate to environmental stresses.

Therefore, numerous kinds of transgenic tobacco plants with valuable traits have been created. The gene encoding for the δ-endotoxin of *Bacillus thuringiensis* (Bt) had been successfully expressed in transgenic tobacco plants and proved to be effective in controlling lepidopteran insects pests (Vaeck *et al*., 1987). Efforts have been made to obtain a higher expression level in order to increase the effectiveness of this technology. The best insecticidal activity was found in tobacco expressing a truncated Bt endotoxin (Barton *et al.,* 1987). Modifications to the bacterial gene sequence of Bt endotoxin were made it more readily expressible in plants and were efficient to obtain resistance against less sensitive pests (Bhau and Koul, 1998).

The cotton bollworm *Heliothis armigera* feeds on at least 120 cultivated plants, included tobacco. Larvae infest the leaves or buds of tobacco plant and cause serious damage with large holes. Tobacco transplanted fields are in heavily infested areas within 3-5 days. The transformation of Bt insecticidal gene *Cry 1Ac* from the *B. thuringiensis* to tobacco plant allows crop protection from lepidopteran insect attack such as *H. armigera.*

For many different plant species, it is possible to obtain transgenic plants using *Agrobacterium*-mediated DNA transformation. Genetics transformation in tobacco has been extensively performed by the *Agrobacterium*-mediated system. The co-cultivation of leaf-discs with *Agrobacterium* can produce tobacco transformants with high quality and fertility.

This paper describes the transformation of tobacco by using *Agrobacterium tumefaciens* and the examination of transgenes in plant genome, especially insect resistant gene *Cry 1Ac*. We observed that the insecticidal crystal protein of the Cry1Ac type was synthesized in Bt tobacco plants and was toxic to feeding larvae of *H. armigera.*

Materials and Methods

Plant material

Tobacco K326 variety was supplied from Tobacco Research Institute (Vietnam). Leaf discs 1x1 cm derived from seed culture were prepared and placed on MS medium supplemented with NAA 0.1 mg/l, at 10/14 light/dark, at 25^{o}C.

Bacterial strain and plasmid

Agrobacterium strain EHA 105 harboring pITB2 vector was used for transformation. PITB2 is derived from plasmid pCAMBIA 3301 (Australia). The T-DNA region of plasmid includes a herbicide resistance gene encoding enzyme to detoxify respective herbicide (*bar* gene), an insect-resistance gene encoding the δ-endotoxin of *B. thuringiensis* (*Cry1Ac* gene) and a β-glucuronidase gene (*gusA* gene).

Infection with *Agrobacterium*

After 2 days of incubation, tobacco cells were infected with *Agrobacterium*. Co-cultivation was carried out at 25°C in the dark on MS medium supplemented with NAA 0.1 mg/l, BA 0.5 mg/l for 2 days. Bacteria were then removed by several washes in Cefotaxim solution (600 mg/l) .

Selection and regeneration of transformants

The infected leaves were subcultured on MS medium containing Cefotaxim 500 mg/l, NAA 0.1 mg/l and BA 1 mg/l for 4 days prior to selection of transferred plates on MS containing PPT 5-10 mg/l.

After at least 2 rounds of selection (3 weeks per round), plates were transferred to MS regeneration medium supplemented with NAA 0.1 mg/l, BA 1 mg/l.

After 20 days of culture, the regenerated shoots were transferred to MS medium containing 10-30 mg/l PPT for the selection of transformed plants. Subsequent culture of 2 cm long shoots in medium without growth regulators led to rooting and plantlet development.

Assay for GUS activity

Tobacco calli and shoots were stained in a phosphate-buffered solution containing 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) at 37°C for 16 hours. They were bleached by soaking in ethanol overnight. Penetration of leaves by X-gluc was faciliated by soaking them in diethyl ether (3 min) followed by three washes in ethanol.

DNA isolation and PCR analysis

Total DNA was isolated using SDS – DNA extraction method. Leaf material was ground in DNA extraction buffer (0.1M Tris-HCl, 0.5M NaCl, 50 mM EDTA pH 8.0) and SDS 20%. The mixture was incubated at 65°C for 10 min. Phenol/chloroform/ isoamyl alcohol (25:24:1) mix was then added. The aqueous phase was separated by centrifugation at 3000 rpm for 5 min. Collect the supernatant and isopropanol was added. After 30 min at 4°C, the mixture was centrifuged at 3,000 rpm for 5 min. The pellet was washed in 70% ethanol and after drying, it was dissolved in TE buffer. This solution was treated with RNAse to make it RNA-free.

Reaction was carried out in 50 μl volume containing 50mM KCl, 10mM Tris-HCl, 1.5 mM $MgCl_2$, 0.2 mM each of four dNTPs, 50 ng each of primers, 1.5 unit *Taq polimerase*, and genomic DNA. For *Cry1Ac* gene, amplification consisted of start of the reaction at 95°C for 7 min and 30 cycles of 1 min at 95°C, 1 min at 54°C and 1 min at 72°C followed by 7 min at 72°C. The sequence of the primers which decribed as the following were designed to amplify 0.605 kb fragment of *Cry1Ac* gene.

1Ac-1: 5'ACAGAAGACCCTTCAATATA 3'

1Ac-2: 5'GTTACCGAGTGAAGATGTAA 3'

Detection of Cry1Ac protein

To confirm *Cry1Ac* gene expression in leaf tissue, Western Blot analysis was done. Fresh leaf tissue was homogenized in extraction buffer (0.125 M Tris-HCl, 4% SDS, 20% glycerol, 0.2 M DTT, 0.02 bromophenol blue, pH 6.8). Total soluble protein was loaded and electrophoresed in a 10% SDS-PAGE gel and then transferred to a nylon membrane and incubated with Cry1Ab polyclonal antibodies (1:3,000 dilution). This antibody was found equally reactive to Cry1Ab and Cry1Ac (Sardana *et al.,* 1996, Cheng *et al.,* 1998). Alkaline phosphatase-conjugated goat anti-rabbit IgG (Pierce) was used (1:5,000 dilution).

Insect bioassay

Young, fresh leaves of transgenic tobacco plants grown in the greenhouse were placed on moist cotton in Petri dishes. A one third-instar larva of *H. armigera* was placed in each plate in 10 replicates for each potted plant. All the Petri dishes were kept under the conditions that were optimal for survival and growth of the insects.

Mortality of larvae and body weight of the individual insects were recorded after 5 days.

Results

Transformation of tobacco

Results from our experiments showed that 10 mg/l PPT were sufficient to suppress shoot regeneration from the control explants. After 3 – 5 weeks of selection on 5-10 mg/l PPT, small shoot primordial were regenerated on the explants co - cultured with EHA 105 (pITB2). After 4 weeks of culture, resistant shoots were developed on 10-30 mg/l PPT medium (Figure 1, Table 1).

(a) (b)

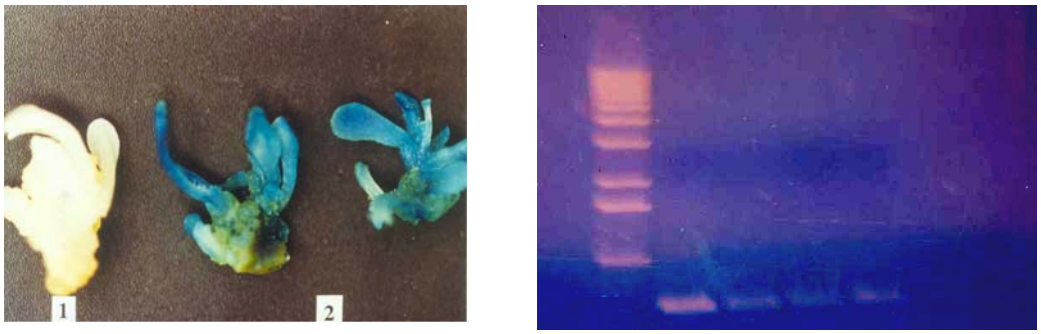
Figure 1 (a) Transformed tobacco shoots regenerated from the explants on PPT medium after 3 weeks of selection and untransformed control.
(b) Tobacco transformants and untransformants on PPT medium after 4 weeks of selection.

Table 1 Percentage of regenerated tobacco explants on PPT medium (%).

Duration of culture	PPT (mg/l)	Transgenic explants			Control		
		No. of original explants	No. of regenerated explants	% of regenerated explants	No. of original explants	No. of regenerated explants	% of regenerated explants
3 weeks	5	20	12	60	20	2	10
5 weeks	10	12	12	100	2	0	0

Integration of T-DNA in the tobacco genome

The PPT-resistant regenerated calli and shoots were examined for GUS activity by histochemical assay. These lines exhibited the high frequency of GUS expression. On average, 60-70% of the transformants were blue in the GUS assay (Figure 2a, Table 2). Subsequently, the interest transgenes were screened by using PCR. The 0.605 kb band on the agarose gels determined the presence of *Cry1Ac* gene in the genome of the initially putative transformants, respectively (Figure 2b).



(a) (b)

Figure 2 (a) Expression of GUS in the transformed tobacco shoots (2) and untransformed control (1)
(b) PCR analysis of tobacco transformants
1: standard DNA
2: DNA from plasmid
3, 4, 5: DNA from transformants
6: DNA from untransformant

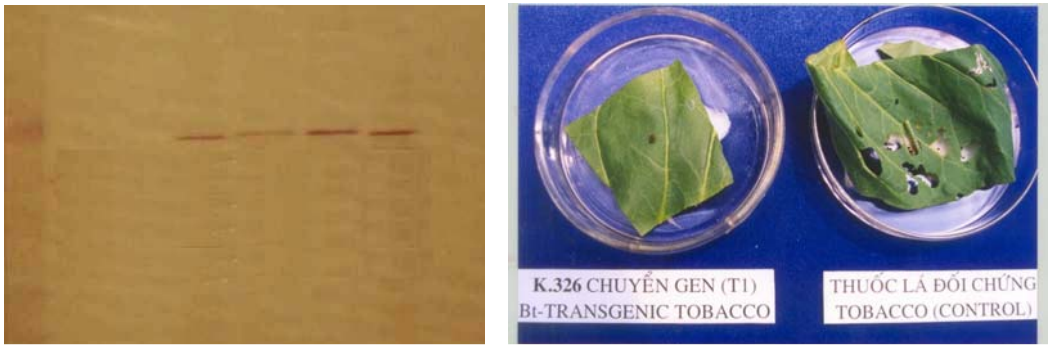
Table 2 Expression of GUS in tobacco shoots after 3 weeks on selection medium.

	Transgenic shoots	Control
No. of original shoots	10	10
No. of blue shoots	10	0
% of blue shoots	100	0

The expressions of *bar* gene and *Cry1Ac* gene

After four weeks growing in the selective medium at the concentration of 30 mg/l PPT, the seedlings derived from the transgenic lines survived and grew as vigorously as the plantlets grown in fresh MS medium. Whereas, all non-transgenic plantlets turned yellow and died (Table 3). These transgenic-surviving plantlets as well as the untransformed ones in the MS medium were removed to plant in pots in the greenhouse (Figure 3b).

Protein encoded by *Cry1Ac* gene in the transgenic lines was examined immunologically by using a polyclonal antibody against Cry1Ab, which also was found to be cross-reactive with Cry1Ac. A major band was seen at the site of 50 kD in the lanes loading the protein extracted from the transgenic plants. No antibody reactive protein was found in the lane of negative control (Figure 3a).



1 2 3 4 5 6

(a) (b)

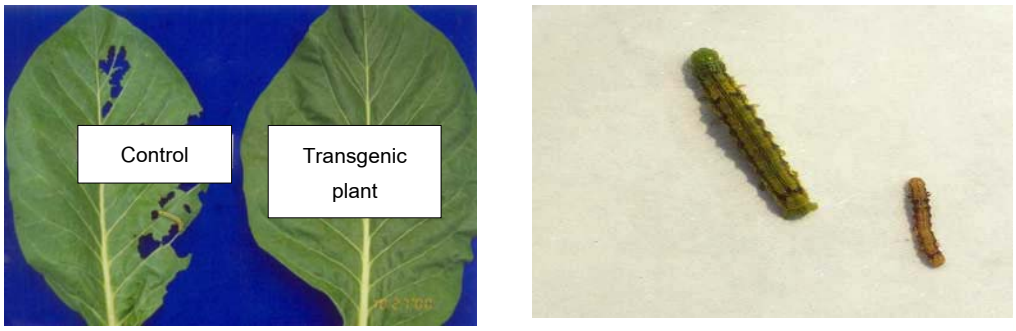
Figure 3 (a) Western Blot of tobacco transformants
1: standard protein
2: protein from untransformant
3, 4, 5, 6: protein from transformants
(b) Control plant was damaged within 5 days

Table 3 Percentage of survived tobacco plantlets on PPT medium after 4 weeks of culture (%).

PPT (mg/l)	Transgenic plantlets			Control		
	No. of plantlets	No. of Survived plantlets	% of Survived plantlets	No. of plantlets	No. of survived plantlets	% of survived plantlets
10	20	20	100	20	0	0
20	20	20	100	20	0	0
30	20	20	100	20	0	0

Insect bioassay

The insecticidal activity of the transgenic tobacco plants was tested in two-month old plants using *H. armigera*. The unstransformed plants were used as control. Since the larvae of *H. armigera* can attack each other, one third-instar larva was placed on each leaf-disc derived from each transgenic plant for 10 replicates. Within 60 hours, the larvae on the transgenic leaves almost stopped feeding and began to be killed (Figure 4a). Five days after feeding, in the genetic engineered lines, the mortality of the insects reached 75% (Table 4). The surviving larvae fed with plants carrying *Bt* gene increased the body weight so much less than of the larvae on the control (Figure 4b). Some larvae were scored to be reduced the body weight. Those transgenic leaf-discs exhibited only minor damage. Whereas, larvae fed on untransformed tissue grew well with mortality of 0% and the increase of the body weight ranging from 8.3 – 8.5 fold (Table 5). These larvae caused severe damage on the untransformed leaves.



(a) (b)

Figure 4 (a) Transformed plant was well protected from leaf damage
(b) *H. armigera* fed on transformants (right) and untransformants (left)

Table 4 Bioassay of *Heliothis armigera* on tobacco leaf of transformed plants.

	Transgenic plants		Control	
	After 3 days	After 5 days	After 3 days	After 5 days
No. of larvae tested	120	120	120	120
No. of larvae died	54	90	0	0
Mortality (%)	45.0	75.0	0.0	0.0

Table 5 Weight increase of *Heliothis armigera* surviving larvae.

	Transgenic plants	Control
Weight of larvae before tested (mg)	12.6	13.0
Weight of larvae 5 days after feeding (mg)	20.3	121.8
% of weight increase (%)	61.1	836.9

Discussion

The results presented in this paper confirm the ability to obtain the transgenic plants using *Agrobacterium*-mediated transformation. It is true that *Agrobacterium* system does not always enable to result in success for any species, especially the monocot varieties. However, at present and in the near future, under the conditions of most agronomic research laboratories in Vietnam, *Agrobacterium*-mediated system is very likely to be the best choice to improve the crops carrying the new agronomically important traits without requiring as much of time as the traditional breeding.

For the pesticide management in agriculture, Bt biopesticide has been used for nearly four decades but the percentage is estimated to be less than 1% worldwide (Krattiger, 1997) because of many restrictions such as: necessity of repeated applications several times per season, breaking down the active ingredient by sunlight, being washed by rain or dew.

Molecular analyses and insect feeding assays revealed that the gene *Cry 1Ac* encoding for the δ-endotoxin of *B. thuringiensis* was successfully expressed in transgenic tobacco plants and proved to be effective in controlling insect pests. The bioassay demonstrated the effectiveness in protecting the transgenic plants from the damage caused by insect as soon as the attack happened. This result confirms that Cry1Ac protein is highly toxic to *H. armigera* larvae. Some studies before also showed that the insecticidal crystal protein of the Cry 1Ac type was effective to lessen the damage of *H. armigera* (Krattiger,1997). Cry1Ac protein from transgenic rice plant is very sensitive to the feeding neonates of *H. armigera* when mixed this toxin with the synthetic medium (Nayak *et al*., 1997).

Therefore, plants containing Bt insecticidal protein indicated the application of plant genetic engineering for the protection of crops against insect attack and opened the door for rapid crop improvement.

Conclusion

Tobacco K326 variety transformed with the Bt genes expressed for the *Cry1Ac* encoding for the δ-endotoxin via the *Agrobacterium* mediated gene transfer contain adequate amount of insecticidal protein. We have used the cotton bollworm in the bioassay by feeding the third-instar larvae feeding on the tobacco carried Bt gene leaf discs for 60 hours. The results revealed that the larvae stopped feeding and death after 5 days of feeding at the rate of 75%. Furthermore, the survived larvae also significantly decreased in body weight than the control ones fed on the untransformed tobacco leaf discs. Our results

should be concretely confirmed the advantages of using the Bt transgenic plants for the crop protection especially against the lepidopterans.

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References

Barton, K.A., H.R. Whiteley and N.S. Yang. 1987. *Bacillus thuringinesis* delta-endotoxin expressed in transgenic *Nicotiana tobacum* provides resistance to lepidopteran insects. Plant Physiol. 85(4): 1103-1109.

Bhau, B.S. and V. Koul. 1998. Switching on *Bacillus thuringiensis* to reduce selection for resistance. Current Science 75(8): 771-777.

Cheng X., R. Sardana, H. Kaplan and I. Altosaar. 1998. *Agrobacterium*-transformed rice plants expressing synthetic *cry1A(b)* and *cry1A(c)* genes are highly toxic to striped stem borer and yellow stem borer. Proc. Natl. Acad. Sci. USA. 95(6): 2767–2772.

Krattiger, A.F. 1997. Insect Resistance in Crops: A Case Study of *Bacillus thuringiensis (Bt)* and its Transfer to Developing Countries. ISAAA Briefs No. 2. ISAAA. Ithaca, NY. 42 pp.

Nayak, P., D. Basu, S. Das, A. Basu, D. Ghosh, N. A. Ramakrishnan, M. Ghosh and S. K. Sen. 1997. Transgenic elite *indica* rice plants expressing Cry1Ac δ-endotoxin of *Bacillus thuringiensis* are resistant against yellow stem borer (*Scirpophaga incertulas*) Proc. Natl. Acad. Sci. USA. 94(6): 2111–2116.

Sardana, R., S. Dukiandjiev, M. Giband, X. Cheng, K. Cowan, C. Sauder and I. Altosaar. 1996. Construction and rapid testing of synthetic and modified toxin gene sequences *cry1A(b&c)* by expression in maize endosperm culture. Plant Cell Reports. 15: 677-681.

Vaeck, M., A. Reynaerts, H. Hofte, S. Jansens, M. De Beuckeleer, C. Dean, M. Zabeau, M. van Montagu and J. Leemans 1987. Transgenic plants protected from insect attack. Nature 328: 33-37.